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Effects of bleomycin and antioxidants on the fatty acid profile of testicular cancer cell membranes

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ABSTRACT

Bleomycin is used in chemotherapy regimens for the treatment of patients having testicular germ-cell tumor (TGCT). There is no study in the literature investigating the effects of bleomycin on membrane lipid profile in testicular cancer cells. We investigated membrane fatty acid (FA) profiles isolated, derivatized and analyzed by gas chromatography of NTera-2 testicular cancer cells incubated with bleomycin (Bleo) for 24 h in the absence and presence of N-Acetyl-L-Cysteine (NAC) and curcumin (Cur) as commonly used antioxidant adjuvants. At the same time the MAPK pathway and EGFR levels were followed up. Bleomycin treatment increased significantly saturated fatty acids (SFA) of phospholipids at the expense of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). Bleomycin also led to a significant increase in the trans lipid isomers of oleic and arachidonic acids due to its free radical producing effect. Incubation with bleomycin increased the p38 MAPK and JNK levels and downregulated EGFR pathway. Coincubation of bleomycin with NAC reversed effects caused by bleomycin. Our results highlight the important role of membrane fatty acid remodeling occurring during the use of bleomycin and its concurrent use with antioxidants which can adjuvate the cytotoxic effects of the chemotherapeutic agents.

1. Introduction

Testicular germ cell tumors (TGCTs) are the most frequent malignancy in males between 20 and 45 years of age. The current tumor incidence in the general population is ≈0.005% which is 50% higher in comparison to 30 years ago and the number of diagnosed cases keeps increasing gradually [1], however, the causes of this increase remain unclear [2]. TGCTs represent the majority of testicular tumors (>95%). Men with TGCTs have a higher risk of developing a subsequent tumor and a second primary contralateral testis tumor may occur in up to 5% of men with a prior tumor [3]. Conventional cancer treatments with some therapeutic drugs and radiation might generate free radicals (ROS) which exert additional cytotoxic effects such as induction of apoptosis. Bleomycin is commonly used to treat patients with TGCTs [4]. Bleomycin binds to and cleaves DNA in the presence of ferrous ion and molecular oxygen [5] involving the following three actions: (i) recognition of a particular base or base sequence on a DNA double strand, (ii) formation of radical species that propagates free radical based mechanism of action, and (iii) oxidation reactions leading to DNA strand scission [6]. Bleomycin generates the highest level of free radicals which act as one of the intracellular second messengers leading to induction of various proteins through transcriptional activation [7]. Under these conditions, the unsaturated fatty acid residues of cell membranes may undergo oxidation and isomerization. Indeed, in our recently published study performed in liposomes and cell cultures, we demonstrated that bleomycin–iron complex transformed membrane mono- and polyunsaturated fatty acid components (MUFA and PUFA) into trans geometric isomers together with a profound remodeling of the membrane fatty acid residues [8]. Transformation of cis to trans geometry of unsaturated lipids has been described in other conditions in cells, animal models, as well as in humans [9]. It is worth to underline that trans fatty acid isomers cannot be synthesized in eukaryotic cells, and an enzymatic cis–trans isomerization occurs only in some bacteria [9,10]. The presence and effect of trans fatty acids in dietary foods consumed have been thoroughly evaluated for health [9,11,12]. Antioxidants prevent excess free radical formation and reactions, and exert inhibitory activity in lipid isomerization [13]. Intrinsic antioxidants represent the only way to protect the lipid geometry. There are conflicting views for the use of antioxidants in cancer patients due to their potential interactions with radiation and chemotherapy induced ROS generation [14,15]. Focusing on cell membranes as the essential element of the cells and lipidomic monitoring of the fatty acid residues of membrane

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phospholipids in cell cultures have demonstrated the effect of dietary conditions during the chemotherapeutic treatments. Lipidomic monitoring has been shown to be a powerful tool to follow up the consequence of diet and treatment on membrane fatty acid reorganization [16,17].

In the present study, we aimed to analyze the fatty acid remodeling of human testicular cancer cell membranes (NTera-2) incubated with bleomycin in the absence and presence of antioxidant supplementation of curcumin and NAC. Curcumin is the principal curcuminoid of the popular Indian spice turmeric, which is a member of the ginger family (Zingiberaceae). N-acetylcysteine (NAC) is a pharmaceutical drug used also as a nutritional supplement and is a cysteine source for the synthesis of glutathione. In addition to investigating the lipidomic monitoring, we determined also the EGF receptor, a transmembrane glycoprotein involved in the regulation of cell proliferation, differentiation, and survival; and mitogen-activated protein kinase (MAPK), one of the most important response to oxidative stress, as two important biomarkers of signaling pathways. We searched if the results of lipidome analysis highlight the role of bleomycin induced fatty acid alterations in cancer cell membranes as well as the effect of combined antioxidant supplementation, in order to envisage possible interference or synergism with the effect of bleomycin.

2. Methods

2.1. Cell culture

NTera-2 human testicular germ cancer cells provide a model system for investigating potential mechanisms of testicular cell membrane alterations induced by bleomycin and antioxidants *in vitro*. NTera-2 cells were obtained from the ATCC. We did not detect any infection (mycoplasma or other pathogens) in the NTera-2 cells. According to the material transfer agreement form attached, ATCC guarantees the provision of infection free cells. Additional analysis in our laboratory verified that the cells were infection free. As proposed by the reviewer, we added these sentences in the manuscript. The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin and 100 μg/ml of streptomycin in a 5% CO_2 atmosphere at 37 °C. We incubated the cells with the IC_{50} concentrations of bleomycin (0.28 mM) and curcumin (20 μM) for 24 h [18]. We used 5 mM NAC dose for 24 h incubations [19]. The curcumin was purchased from Sigma-Aldrich (Cat No C7727). The standard protocols for cell culture were applied in all of the experiments and all the cell groups were prepared under the same conditions.

2.2. Phospholipid extraction and fatty acid analysis

Cells were detached using accutase, thoroughly washed with phosphate buffer, and pelleted by centrifugation at 14,000 ×*g* for 40 min at 4 °C after adding water. Phospholipids were isolated from the pellet and fatty acids were derivatized as described [20]. Fatty acid compositions are given in Table 1 as relative percentages of the total fatty acid content.

2.3. Mitogen-activated protein kinase (MAPK) assay

Cells were lysed with Cell Lysis Buffer (9803; Cell Signaling Technology), then the levels of p38, p-MEK1/2, p-ERK1/2, and p-SAPK/JNK in the cell lysates were determined using the PathScan MAPK Multi-Target Sandwich ELISA Kit (7274; Cell Signaling Technology) according to the manufacturer's instructions.

2.4. Epidermal growth factor receptor (EGFR) assay

Epidermal growth factor receptor levels were measured by sandwich ELISA kit (Calbiochem), a sensitive colorimetric assay, in accordance with the procedure recommended by the manufacturer. The total protein content in the samples was determined by the Bradford method [21] using bovine serum albumin (BSA) as a standard. The total protein content was used to normalize the EGFR and MAPK values of each sample.

2.5. Statistical analysis

Results were given as mean ± SD. Statistical comparisons were conducted using t-test and SPSS software; version 13.0 (Chicago, IL). Statistical significance was based on 95% confidence limits ($p \leq 0.05$). Comparison of the non-parametric data among the groups was performed using the Mann–Whitney U test.

3. Results

3.1. Membrane fatty acid profile

Table 1 shows membrane fatty acid composition in the NTera-2 cells after incubations with bleomycin and antioxidants for 24 h. Fig. 1 depicts the main fatty acid changes detected in the NTera-2 cell membrane phospholipids after 24 h exposure to bleomycin, NAC, curcumin, bleomycin + NAC, and bleomycin + curcumin. Palmitic acid (16:0) level was found similar in all of the groups. A significant increase in the stearic acid (18:0) residue was observed in the cells incubated with bleomycin and bleomycin + NAC compared to the control cells, and NAC decreased stearic acid level compared to the cells incubated with bleomycin. Bleomycin + NAC significantly enhanced stearic acid compared to NAC. As shown in Table 1, incubation with bleomycin alone or in combination with NAC or curcumin diminished significantly ($p < 0.05$, $p < 0.01$) the levels of monounsaturated cis-fatty acids; 6c-16:1, 9c-16:1, 9c-18:1 (oleic), and 11c-18:1 (vaccenic). Incubation with bleomycin and its combination with NAC or curcumin decreased palmitoleic acid (9c-16:1) levels compared to the control cells (<0.05). Bleomycin + NAC significantly decreased palmitoleic acid compared to NAC (<0.05). The trans isomers 9t-16:1 and 6t-16:1, expressed as trans-16:1, significantly increased in the cells incubated with bleomycin, curcumin and bleomycin + curcumin compared to the control cells ($p < 0.001$). NAC decreased trans-16:1 compared to bleomycin. Bleomycin + NAC and bleomycin + curcumin increased trans-16:1 compared to NAC. Bleomycin + curcumin significantly increased also the trans-16:1 than curcumin (<0.001). The level of the omega-6 essential fatty acid, linoleic acid (9c,12c-18:2) decreased in all of the cell groups incubated with bleomycin and its combination with the antioxidants compared to the control group ($p < 0.05$). The level of arachidonic acid (20:4) significantly diminished in the cells incubated with bleomycin (58%), bleomycin + NAC (%54), and bleomycin + curcumin (%51) compared to the control cells as shown in Fig. 1. The levels of the precursor of arachidonic acid in the omega-6 pathway, *i.e*. eicosatrienoic (8c,11c,14c-20:3) acid increased in the cells incubated with bleomycin and its combination with curcumin ($p < 0.05$). Fig. 2 depicts the levels of the three fatty acid families; SFA, MUFA and PUFA under the six experimental conditions.

The PUFA percentage significantly decreased in the cells incubated with bleomycin, curcumin, bleomycin + NAC, and bleomycin + curcumin compared to the control as shown in Fig. 2. MUFA percentage significantly decreased and the SFA percentage significantly increased by the combination of bleomycin + NAC compared to NAC alone group (<0.01). A 24 hour incubation with different agents caused significant changes in the membrane fatty acid composition compared to the controls. Incubations with bleomycin, curcumin, bleomycin + NAC and bleomycin + curcumin increased SFA/MUFA ratios significantly. In contrast, incubation with NAC alone did not cause any change in the membrane fatty acid family compositions as well as in SFA/MUFA ratios compared to the control. The crucial modifications in the membrane fatty acid composition were observed following incubations with